

LB-antibiotic liquid medium on a rotary shaker (250 rpm) at 37°, using procedures recommended in the pET System Manual to maximize pET plasmid retention. At an OD₅₂₅ of 2.4, cells from this 200 ml culture were collected by centrifugation and resuspended in 50 ml of fresh medium. This suspension was transferred to a high density fermentor containing 4 liters of carbenicillin- and chloramphenicol-containing SLBH medium (the composition of SLBH medium, and the design and operation of the fermentor are described in (Sadler et al. 1974)). After 20 hours of growth under O₂ at 32° (OD₅₂₅ = 19) isopropylthiogalactoside (IPTG) was added to 0.4 mM to induce uricase production. After 6 more hours (OD₅₂₅ = 37) bacterial cells were harvested by centrifugation (10,410 x g, 10 min, 4°C), washed once with PBS, and stored frozen at -20°C.

The bacterial cells (189 g) were resuspended in 200 ml PBS and lysed while cooled in an ice/salt bath by sonication (Heat Systems Sonicator XL, probe model CL, Farmingdale, NY) for 4 x 40 second bursts at 100% intensity, with a 1 minute rest between bursts. PBS-insoluble material (which includes uricase) was pelleted by centrifugation (10,410 x g, 10 min, 4°C), and was then washed 5 times with 200 ml PBS. Uricase in the PBS-insoluble pellet was extracted into 80 ml of 1 M Na₂CO₃, pH 10.2 containing 1 mM phenylmethylsulfonylfluoride (PMSF) and 130 µg/ml aprotinin. Insoluble debris was removed by centrifugation (20,190 x g, 2 hours, 4°C). All further steps in purification were at 4°C (results summarized in Table 3).

The pH 10.2 extract was diluted to 1800 ml with 1 mM PMSF (to reduce Na₂CO₃ to 0.075 M). This was applied to a column (2.6 x 9 cm) of fresh Q-Sepharose (Pharmacia Biotech, Inc., Piscataway, NJ), which had been equilibrated with 0.075 M Na₂CO₃, pH 10.2. After loading, the column was washed successively with 1) 0.075 M Na₂CO₃, pH 10.2 until A₂₈₀ absorbance of the effluent reached background; 2) 10 mM NaHCO₃, pH 8.5 until the effluent pH fell to 8.5; 3) 50 ml of 10 mM NaHCO₃, pH 8.5, 0.15 M NaCl; 4) a 100-ml gradient of 0.15 M to 1.5 M NaCl in 10 mM NaHCO₃, pH 8.5; 5) 150 ml of 10 mM NaHCO₃, pH 8.5, 1.5 M NaCl; 6) 10 mM NaHCO₃, pH 8.5; 7) 0.1 M Na₂CO₃, pH 11 until the effluent pH was raised to 11. Finally, uricase was eluted with a 500 ml gradient from 0 to 0.6 M NaCl in 0.1 M Na₂CO₃, pH 11. The activity eluted in two A₂₈₀-absorbing peaks, which were pooled separately (Fraction A and

Fraction B, Table 3). Uricase in each of these pools was then precipitated by lowering the pH to 7.1 by slow addition of 1 M acetic acid, followed by centrifugation (7,000 x g, 10 min). The resulting pellets were dissolved in 50 ml of 1 M Na₂CO₃, pH 10.2 and stored at 4°C.

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Table 3

Recombinant Pig-Baboon Chimeric (PBC) Uricase Purification

IPTG-induced Cell Paste = 189.6 g

Fraction	Total Protein mg	Uricase activity U/ml	Total Uricase Units	Specific Activity U/mg
pH 7 Sonicate + pH 7 Wash			74.9	
pH 10.2 Extract	4712	82.7	11,170	2.4
Q-Sepharose fraction A	820	11.5	1,081*	1.9
fraction B	1809	31.7	4,080	2.3
pH 7.1 precipitated & redissolved fraction A	598	35.0	1,748	3.0
fraction B	1586	75.5	3,773	2.4
Total Recovery	2184		5,521	

10 *The uricase present in fraction A began to precipitate spontaneously after elution from the column. Therefore activity measured at this stage of purification was underestimated.

EXAMPLE 3

Small scale preparation and PEGylation of recombinant PBC uricase.

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This example shows that purified recombinant PBC uricase can be used to produce a PEGylated uricase. In this reaction, all uricase subunits were modified (Figure 1, lane 7), with retention of about 60% of catalytic activity (Table 4).

10 A. Small scale expression and isolation of PBC uricase (Table 4, Figure 1).

A 4-liter culture of *E.coli* BL21(DE3)pLysS transformed with pET3d-PBC cDNA was incubated on a rotary shaker (250 rpm) at 37°. At 0.7 OD₅₂₅, the culture was induced with IPTG (0.4 mM, 6 hours). The cells were harvested and frozen at -20°C.

15 The cells (15.3 g) were disrupted by freezing and thawing, and extracted with 1 M Na₂CO₃, pH 10.2, 1 mM PMSF. After centrifugation (12,000 x g, 10 min, 4°C) the supernatant (85 ml) was diluted 1:10 with water and then chromatographed on Q-Sepharose in a manner similar to that described in Example 1. Pooled uricase activity from this step was concentrated by pressure ultrafiltration using a PM30 membrane
20 (Amicon, Beverly, MA). The concentrate was chromatographed on a column (2.5 x 100 cm) of Sephacryl S-200 (Pharmacia Biotech, Piscataway, NJ) that was equilibrated and run in 0.1 M Na₂CO₃, pH 10.2. Fractions containing uricase activity were pooled and concentrated by pressure ultrafiltration, as above.

25 B. PEGylation.

100 mg of concentrated Sepahacryl S-200 PBC uricase (5 mg/ml, 2.9 µmol enzyme; 84.1 µmol lysine) in 0.1 M Na₂CO₃, pH 10.2 was allowed to react with a 2-fold excess (mol of PEG:mol uricase lysines) of an activated form of PEG at 4° for 60 min.

30 The PEGylated uricase was freed from any unreacted or hydrolyzed PEG by tangential flow diafiltration. In this step the reaction was diluted 1:10 in 0.1 M Na₂CO₃, pH 10.2